AD_____

Award Number: DAMD17-97-1-7301

TITLE: Development of a Novel, Proteinase-Activated Toxin
Targeting Tumor Neovascularization

PRINCIPAL INVESTIGATOR: Rodney K. Tweten, Ph.D.

CONTRACTING ORGANIZATION: University of Oklahoma
Oklahoma City, Oklahoma 73104

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (30 Sep 98 - 29 Sep 99)

4. TITLE AND SUBTITLE
Development of a Novel, Proteinase-Activated Toxin Targeting Tumor Neovascularization

5. FUNDING NUMBERS
DAMD17-97-1-7301

6. AUTHOR(S)
Rodney K. Tweten, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma
Oklahoma City, Oklahoma 73104

E-MAIL:
rodney-tweten@ouhsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research is to target *Clostridium septicum* alpha toxin to the cells in blood vessels that are undergoing angiogenesis durin there invasion of tumors. This will be accomplished by altering the normal proteolytic activation site of alpha toxin to one which is activated by gelatinase enzymes which are primarily active in this endothelial cells. The presentation of the gelatinase cleavage in alpha toxin appeared to be problematic since we were unable to generate mutants of alpha toxin with the normal furin activation site replaced with a gelatinase A and B that could be efficiently activated with gelatinase B. It was clear that when alpha toxin was largely misfolded and inactive that the engineered gelatinase site could be efficiently cleaved by gelatinase B. Therefore, in the light of this information and new information that we and others have generated with respect to gelatinase activity and the structure-function relationships of alpha toxin we are pursuing several different approaches to solve the gelatinase activation problem of alpha toxin. We have also begun the application of our approach to other cytolytic toxins which may be more amenable to the engineering of gelatinase switches into their structures so that they can be targeted them to blood vessels which are feeding tumors.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Table of contents

A. Introduction

This work herein describes the initial stages in the development of a unique and innovative therapy for eliminating tumors. Unlike other tumor therapies in which the tumor itself is the primary target, the gelatinase-activated toxins to be developed in this study will primarily impact tumor vasculature, but with the added benefit of attacking invading tumor cells. Since tumor types vary widely in their susceptibility to cancer therapies, treatments based on tumor cell recognition often lead to the development of resistant tumor cells. This is particularly true when the treatment consists of drugs that are susceptible to cellular detoxifying mechanisms. Other therapies based on immunological approaches often require that a unique target be identified on the tumor cell that distinguishes it from a normal cell. The search for tumor-specific markers has been difficult since often times it is not a question of the absence or presence of a marker, but the relative concentration of the marker on tumor cells versus the level on normal cells. In the approach proposed here, we bypass the need to target a unique aspect of the tumor itself, and target the neovascularization of the tumor. The process of angiogenesis (the production of new capillaries) is necessary for the growth of breast tumors and other solid tumors. Targeting the angiogenic process enables us to target a wide variety of tumor types rather than just a single or few tumor types. In addition, tumors cannot become resistant to this form of therapy since it is not a tumor cell antigen or receptor that is the target. A fundamental requirement for angiogenesis is the dissolution of basement membranes, and possibly other extracellular matrix proteins, by the action of metalloproteinases. The fact that MMP inhibitors are such potent anti-angiogenic agents strongly supports this model. We are in a unique position to target the neovascularization of breast tumors for the following reasons: 1) we work on a unique Clostridial toxin which has a high cytolytic activity towards the vascular endothelium, 2) the toxin requires proteolytic activation, and 3) it appears that we can significantly alter the protease activation site of this toxin without affecting its structure or activity.

Neovascularization of tumors requires the localized presence and activation of MMPs, and most likely gelatinase A, to break down the basement membrane for the development of new capillary vessels. Therefore it is possible that we can target this process by the generation of gelatinase A target sequences in the activation site of *Clostridium septicum* alpha toxin. We have also proposed to develop more general, pan-MMP activated toxins with optimal substrate characteristics for cleavage by gelatinase A, but with some susceptibility to other MMPs. Based on studies with broad specificity MMP inhibitors used in several animal models, and based on a large number of localization studies, MMPs are typically not expressed in normal tissues, and, when expressed, appear to be latent. In other words, MMP activity is highly controlled, and occurs during developmental and repair processes, as well as pathologically. The few tissues known to express MMPs normally, including cycling uterus, may be susceptible to MMP-activated toxins. Because the mechanisms utilized by tumor cells to invade and attract a vasculature are common to those used by normal tissues, the problem of absolute tumor specificity is shared by all therapies. We believe that the toxins to be developed in this study offer a unique and effective means of combating tumor growth. It combines the specificity and efficacy of targeting active MMPs with the lethality of a very potent toxin. Treatments would thus be rapid, eliminating the need for a lifetime of therapy with its attendant complications and expense.

B. Body

1. Abbreviations and nomenclature used in this report

AT^{pro}: Native protoxin, protoxin is is the inactive form of alpha toxin and requires proteolytic activation by normal cell proteases such as furin in order to be converted to the cytolytically active toxin, AT^{act}.

$AT^{PLGIAG396}$: Alpha toxin in which the native sequence of AT^{pro} starting with residue 396 has been replaced by genetic engineering to now code for PLGIAG. Thus, any toxin designated with an superscripted amino acid

sequence followed by a number describes a mutated alpha toxin in which the residues in the superscript replace the residues of the native sequence start with that numbered amino acid in the superscript.

Gelatinase A and B (Gel A and Gel B, respectively): These matrix metalloproteinases have been purified and pre-activated for the *in vitro* assays of there cleavage activity towards the various recombinant forms of alpha toxin which contain consensus gelatinase cleavage sites.

PFO: perfringolysin O, a cholesterol-dependent toxin produced by *Clostridium perfringens*.

IPTG: isopropylthiogalactoside, a gratuitous inducer of the *Tac* promoter.

2. *Overview of the year 2 effort towards re-engineering alpha toxin to selectively target the process of angiogenesis*

Our primary efforts in the second year of this grant have been a continued focus on the generation of additional substrate sites for the gelatinase A and B proteinases which replace the normal furin activation site of alpha toxin. Thus far we have tested several variations on the positioning of the PLGIAG site within the region of alpha toxin which requires proteolytic activation (residues 390-410). However, we have discovered that the presentation of the gelatinase cleavage sequence is apparently not optimal in the alpha toxin mutants we have engineered thus far. This conclusion is based on several independent results. The first of these results was reported last year but the full impact of those results were not appreciated until now. This result concerned the expression of alpha toxin as a fusion protein with glutathione-S-transferase (see Fig. 1) One of the mutants generated in the first year, which carried the PLGIAG site at position 398 in the alpha toxin sequence, was expressed in a system that we have not used previously for the expression of alpha toxin. In spite of good yields of the recombinant toxins when expressed as a fusion in pGEX 4T-2 we originally found that alpha toxin derived by this means is only 10-20% as active as active as native toxin or the recombinant native toxin expressed in the pET22b+ system. We now know that most of the toxin derived by this method must be largely folded incorrectly. The incorrect folding led to the ultimate discovery that this toxin was efficiently cleaved by the gelatinase B (near 90%). But since it retains very little activity the data suggests that the only toxin molecules that are cleaved are those which are folded improperly. Although this molecule is useless for the proposed work it does offer us a convenient assay to make sure our gelatinase enzymes are active in vitro.

Fig. 1. Expression of an glutathione-s-transferase-alpha toxin fusion protein containing the PLGIAG gelatinase B site at position 398. The vector pGEX 4T-2 fused the alpha toxin directly to glutathione-S-transferase which resulted in the expression of the fusion protein in the cytoplasm of *E. coli*. pGEX 4T-2 is driven by the *tac* promoter and is induced with the addition of IPTG to the culture.

We have continued in the second year to generate more PLGIAG and PQGIAG cleavage sites at various locations within the activation site of alpha toxin in the hope that one of these toxins will express a more accessible activation site. The production of these mutants has been time consuming since we must generate the mutant, express it in *Escherichia coli* and purify it for analysis. Thus far we have determined that the PLGIAG sequence located at positions 396, 398 and 402 in the alpha toxin sequence have either not been cleaved or cleave relatively poorly (10-20%) with toxin:gelatinase B ratios of 20:1. In addition, theses same mutants have only a 2-4 fold selectivity for killing cells that express gelatinase B over those which don□t expressgelatinase B.

These three mutants span the the cleavage site of alpha toxin as shown in Fig. 2. The fact that these three mutants all exhibited poor activation and discrimination between Gel^+ and Gel^- cell types suggests that presentation of the cleavage site is not optimal.

```
Native AT        388-PLPDKKRRGKRSVDSLDARLQNEGIRIENI

PLGIAG396                        PLGIAG
PLGIAG398                          PLGIAG
PLGIAG402                              PLGIAG
```

Fig. 2. Location of the PLGIAG 396, 398 and 402 mutants of the alpha toxin cleavage site.

At the end of April I also lost the technician for this project, Lori Bentsen, who carried out much of the work of the first and part of the second year (her husband moved to take a job in Pennsylvania). The loss of Lori had a significant impact on the project since we were required to find and hire her replacement (Karen Ballard) During the second year we have had to replace Lori and train in a new research technician which required several months to accomplish. As of midsummer we had hired another permanent technician to replace Lori and have now trained her sufficiently to continue the project. However, as it turns out Lori Bentsen will also be returning to the lab in late December, 1999 and thus will greatly facilitate our work on this project. We hope to continue the work on the mutants we have already generated but also to expand our search for ways to improve our targeting of the toxin based on the gelatinase activity of the tumor vasculature.

During the training of Karen Ballard, our new technician for this project, we also had her begin the exploration of an alternative approach to gelatinase activation of another cytolytic toxin, perfringolysin O (PFO). The premise of these experiments is that we believe that by adding a large heterologous protein to either the amino or carboxy terminal end of PFO we could produce an inactive toxin that would require the cleavage of the heterologous protein (in this case glutathione-S-transferase or GST) to restore the cytolytic activity of PFO. Initially we made the fusion between PFO and GST with the GST at the carboxy terminus of PFO (Fig. 2, lower figure) and engineered in a gelatinase cleavage site between the two proteins. We were unable to cleave this fusion protein with gelatinase B and therefore did not regain activity of the PFO. However, this was just the first attempt at generating a proteolytic activated PFO and we are continuing with this effort. We are particularly interested in altering the cleavage site so that it is flanked by cysteine residues so that they form a disulfide and loop out the cleavage site (described below in part 3, future directions). This possibility is based on the recent work of Koivunen et al. (Koivunen et al., 1999) who demonstrated that inhibitor peptides of gelatinase A and B were significantly more effective at inhibiting the proteinases if the peptide was looped out by the presence of a disulfide bridge that linked the two ends of the inhibitor peptide. We also made the amino terminal fusion between PFO and GST (Fig. 3, upper figure) but discovered that the toxin remained active with the GST fused to the carboxy terminus. Therefore the amino terminal fusion will not work and we will continue to concentrate on the carboxy terminal fusion to determine if we can generate a gelatinase-cleavable derivative of the PFO-GST fusion protein that restores the cytolytic activity of the PFO.

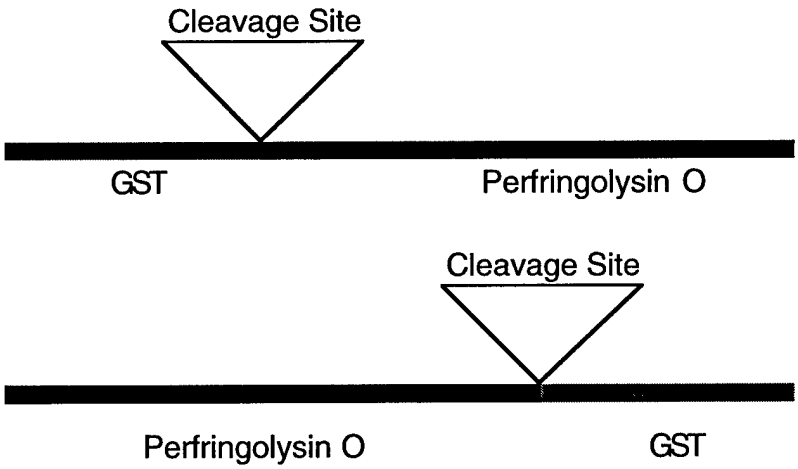

Fig. 3. Fusions of Perfringolysin O with glutathione-S-transferase (GST). The PLGIAG cleavage site is ptoposed to replace the existing thrombin cleavage site which already exists in this construct (denoted as the cleavage site in the figure)

3. *Future Directions for the next (third) year*

a. Generation of cysteine looped cleavage sites for gelatinase in alpha toxin

Recently Koivunen et al. (Koivunen et al., 1999) demonstrated that the peptide CTTHWGFTLC specifically binds to gelatinases A and B, but is not cleaved. Therefore it specifically targets angiogenic tissue. The work of Koivunen et al. (Koivunen et al., 1999) has provided us a new path for constructing alpha toxin derivatives that can be specifically activated by gelatinase B. As described above in the results section we are convinced that the display of the gelatinase substrate sequences in the alpha toxin activation site is not optimal for gelatinase B cleavage. This may be overcome by the looping out of the this cleavage region by the addition of cysteine residues at the ends of the recognition sequence or possibly by the introduction of specific flanking residues that result in the display of the sequence more effectively. It was clear from the data of Koivunen et al. (Koivunen et al., 1999) that the inhibitor sequences required the presence of a disulfide loop to properly display the inhibitor sequence. Therefore, we are currently engineering the gelatinase B PLGIAG cleavage site in alpha toxin with flanking cysteines to loop it out for better display and hopefully better recognition by the gelatinase A and B enzymes.

b. Generation of gelatinase targeting of an alpha toxin receptor binding deficient mutant

The work of Koivunen et al. (Koivunen et al., 1999) also provides us a new and exciting possibility expand the approaches to target alpha toxin to blood vessels undergoing angiogenesis. We have now discovered the receptor binding site of alpha toxin and can specifically knockout receptor function of the toxin in a way that requires only a single amino acid change and which eliminates 100% of the activity. The mutated toxin can still be activated in solution and can still form oligomers in solution but remains inactive on cells. The fact that the receptor function is completely abolished by one mutation is somewhat dramatic and is what makes our opportunity to replace the receptor function with one of our choice relatively unique. We have also found that the receptor function can be partially restored by the addition of other receptor binding sites to the amino terminus of alpha toxin. Hence, we have a rare opportunity to replace the normal receptor function of alpha toxin with one that specifically targets it to angiogenic tissue.

We therefore propose to engineer in the CTTHWGFTLC sequence, which specifically binds to active gelatinase, into various sites of the receptor deficient mutant of alpha toxin. Presumably this will bind the receptor deficient mutant of alpha toxin to only gelatinase producing cells. We will then test these mutants for the ability to bind to gelatinase A and B and to specifically kill gelatinase producing cells. Since the normal activation site will be intact the toxin should be activated normally and thus kill the gelatinase expressing cells. We feel strongly that this alternative approach to target alpha toxin to the blood vessels that feed tumors holds significant potential and we are the only ones in the field of toxin biology curerently able to take advantage of it.

c. Engineering a gelatinase-activated PFO gene

We also intend to pursue the development of a proteolytically (specifically gelatinase) activated derivative of PFO. For many years we have also studied the mechanism of perfringolysin O, one of the cholesterol-dependent cytolysins, and considered the possibility of creating a proteolytically activated derivative of this toxin. We understand the mechanism of cytolysis by this family of toxins very well (Rossjohn et al., 1997; Shatursky et al., 1999; Shepard et al., 1998) and thus believe that we can engineer in a gelatinase-activated switch in this toxin.

C. Key research accomplishments

- **Identification of problems associated with the specificity of the gelatinase B for it s cleavage site**
- **Identification of alternative approaches to improve specificity of the proteolytic activation process**
- **alternative approaches are proposed to specifically target alpha toxin to tumor neovascularization sites based on the specificity of a recently developed angiogenic inhibitor (Koivunen et al., 1999).**
- **Initiation of experiments to expand our concept of engineering a gelatinase activated cytolytic toxin to perfringolysin O**

D. REPORTABLE OUTCOMES

At this time there are no reportable outcomes at this time due to the exploratory nature of this research.

E. CONCLUSIONS

In the first year we generated many of the mutants of alpha toxin which were initially tested for specificity of gelatinase activation. However, as was reported here in the second year we were not able to improve the specificity of the alpha toxin mutants over that which was initially reported. However, with the new information recently reported on the structure of gelatinase inhibitors we feel renewed confidence that we may be able to achieve our goal of specifically targeting angiogenesis with the ultimate goal of destroying the blood supply to tumors.

We still feel confident that this approach will ultimately work and hope that armed with the new information we can now attack this problem of targeting alpha toxin to tumor neovascularization sites in several ways which are based on targeting toxins to gelatinase expressing cells. We feel strongly that this approach is a viable one that requires much exploratory work since this type of approach has never been suggested or attempted before. If we can achieve even partial success with any of the proposed approaches it is likely that the results of this research may potentially become the basis for the application of additional funding and the development of potent anti-cancer therapeutics. We have only scratched the surface of the possibilities of this approach. I have considerable optimism for the next year since we now know what we can and cannot do and now have new information that will help us in the next stage of development of this potential cancer treatment.

F. References

Koivunen, E., Arap, W., Valtanen, H., Rainisalo, A., Medina, O. P., Heikkila, P., Kantor, C., Gahmberg, C. G., Salo, T., Konttinen, Y. T., Sorsa, T., Ruoslahti, E., and Pasqualini, R. (1999). Tumor targeting with a selective gelatinase inhibitor. Nat Biotechnol *17*, 768-74.

Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K., and Parker, M. W. (1997). Structure of a cholesterol-binding thiol-activated cytolysin and a model of its membrane form. Cell *89*, 685-692.

Shatursky, O., Heuck, A. P., Shepard, L. A., Rossjohn, J., Parker, M. W., Johnson, A. E., and Tweten, R. K. (1999). The Mechanism of Membrane Insertion for a Thiol-Activated Cytolysin: A New Paradigm for Pore-Forming Toxins. Cell *in press*.

Shepard, L. A., Heuck, A. P., Hamman, B. D., Rossjohn, J., Parker, M. W., Ryan, K. R., Johnson, A. E., and Tweten, R. K. (1998). Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin

Clostridium perfringens perfringolysin O: an α-helical to β-sheet transition identified by fluorescence spectroscopy. Biochemistry *37*, 14563-14574.